the other suppressor genes that play a role in the tumorigenesis process. Prominent among these is the one(s) presumptively located at 5q21. Cytogenetic (Herrera et al., Am J. Med. Genet., Vol. 25, p. 473 (1986) and linkage (Leppert et al., Science, Vol. 238, p. 1411 (1987); Bodmer et al., Nature, Vol. 328, p. 614 (1987)) studies have shown that this chromosome region harbors the gene responsible for familial adenomatous polyposis (FAP) and Gardner's Syndrome (GS). FAP is an autosomal-dominant, inherited disease in which affected individuals develop hundreds to thousands of adenomatous polyps, some of which progress to malignancy. GS is a variant of FAP in which desmold tumors, osteomas and other soft tissue tumors occur together with multiple adenomas of the colon and rectum. A less severe form of polyposis has been identified in which only a few (2-40) polyps develop. This condition also is familial and is linked to the same chromosomal markers as FAP and GS (Leppert et al., New England Journal of Medicine, Vol. 322, pp. 904-908, 1990.) Additionally, this chromosomal region is often deleted from the adenomas (Vogelstein et al., N. Engl. J. Med., Vol. 319, p. 525 (1988)) and carcinomas (Vogelstein et al., N. Engl. J. Med., Vol. 319, p. 525 (1988); Solomon et al., Nature, Vol. 328, p. 616 (1987); Sasaki et al., Cancer Research, Vol. 49, p. 4402 (1989); Delattre et al., Lancet, Vol. 2, p. 353 (1989); and Ashton-Rickardt et al., Oncogene, Vol. 4, p. 1169 (1989)) of patients without FAP (sporadic tumors). Thus, a putative suppressor gene on chromosome 5q21 appears to play a role in the early stages of colorectal neoplasia in [beth] both sporadic and familial tumors.

a2

At column 2, line 26:

It is another object of the invention to provide a method of screening ~~for genetic~~ [prodisposition] predisposition to cancer.

a3

At column 6, line 42:

Mismatches, according to the present invention are hybridized nucleic acid duplexes which are not 100% homologous. The lack of total homology may be due to deletions, insertions, inversions, substitutions or frameshift mutations. Mismatch detection can be used to detect point mutations in the gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of tumor samples. An example of a mismatch cleavage technique is the RNase protection method, which is described in detail in Winter et al., Proc. Natl. Acad. Sci. USA, Vol. 82, p. 7575, 1985 and Meyers et al., Science, Vol. 230, p. 1242, 1985. In the practice of the present invention the method involves the use of a labeled riboprobe which is complementary to the human wild-type APC gene coding sequence. The riboprobe and either mRNA or DNA isolated from the tumor tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the riboprobe and the mRNA or DNA. The riboprobe need not be the full length of the APC mRNA or gene but can be a segment of either. [II] If the riboprobe comprises only a segment of the APC mRNA or gene it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

a4

At column 9, line ~~10~~ 21:

According to the present invention a method is also provided of supplying wild-type APC function to a cell which carries mutant APC alleles. Supplying such function should suppress

a5

neoplastic growth of the recipient cells. The wild-type APC gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation the gene will be expressed by the cell from the extrachromosomal location. If a gene portion is introduced and expressed in a cell carrying a mutant APC allele, the gene portion should encode a part of the APC protein which is required for non-neoplastic growth of the cell. More preferred is the situation where the wild-type APC gene or a part of it is introduced into the mutant cell in such a way that it recombines with the endogenous mutant APC gene present in the cell. Such recombination requires a double recombination event which results in the correction of the APC gene mutation. Vectors for introduction of genes [beth] both for recombination and for extrachromosomal maintenance are known in the art and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation, calcium phosphate co-precipitation and viral transduction are known in the art and the choice of method is within the competence of the routineer. Cells transformed with the wild-type [A PC] APC gene can be used as model systems to study cancer remission and drug treatments which promote such remission.

At column 9, line 48:

Similarly, cells and animals which carry a mutant APC allele can be used as model systems to study and test for substances which have potential as therapeutic agents. The cells are typically cultured epithelial cells. These may be isolated from individuals with APC mutations, either somatic or germline. Alternatively, the cell line can be engineered to carry the mutation in the APC allele. After a test substance is applied to the cells, the neoplastically transformed [pheno-type] phenotype of the cell will be determined. Any trait of neoplastically transformed cells can be assessed, including anchorage-independent growth, tumorigenicity in nude mice,

invasiveness of cells, and growth factor dependence. Assays for each of these traits are known in the art.

At column 11, line 54:

This example demonstrates the isolation of a 5.5 Mb region of human DNA linked to the FAP locus. Six genes are identified in this region, all of which are expressed in normal colon cells and in colorectal, lung, [ad] and bladder tumors.

At column 12, line 3:

Three contigs encompassing approximately 4 Mb were contained within the central portion of this region. The [YAC's] YACs constituting these contigs, together with the markers used for their isolation and orientations, are shown in FIG. 1. These YAC contigs were obtained in the following way. To initiate each contig, the sequence of a genomic marker cloned from chromosome 5q21 was determined and used to design primers for PCR. PCR was then carried out on pools of YAC clones distributed in microtiter trays as previously described (Anand et al., Nucleic Acids Research, Vol. 18, p. 1951 (1980)). Individual YAC clones from the positive pools were identified by further PCR or hybridization based assays, and the YAC sizes were determined by PFGE.

At column 12, line 16:

To extend the areas covered by the original YAC clones, "chromosomal walking" was performed. For this purpose, YAC termini were isolated by a PCR based method and sequenced (Riley et al., Nucleic Acids Research, Vol. 18, p. 2887 (1990)). PCR primers based on these sequences were then used to rescreen the YAC library. For example, the sequence from an intron of the FER gene (Hao et al., Mol. Cell. Biol., Vol. 9, p. 1587 (1989)) was used to design PCR primers for isolation of the 28EC1 and 5EH8 YACs. The termini of the 28EC1 YAC were

sequenced to derive markers RHE28 and LHE28, respectively. The sequences of these two markers were then used to isolate YAC clones 15CH12 (from RHE28) and 40CF1 and 29EF1 (from LHE28). These five [YAC's] YACs formed a contig encompassing 1200 kb (contig 1, FIG. 1B).

At column 12, line 41:

Multipoint linkage analysis with the various markers used to define the contigs, combined with PFGE analysis, showed that contigs 1 and 2 were centromeric to contig 3. These contigs were used as tools to orient and/or identify genes which might be responsible for FAP. Six genes were found to lie within this cluster of [YAC's] YACs, as follows:

At column 13, line 1:

Contig 2: TB1--TB1 was identified through a cross-hybridization approach. Exons of genes are often evolutionarily conserved while introns and [intergenie] intergenic regions are much less conserved. Thus, it a human probe cross-hybridizes strongly to the DNA from non-primate species, there is a reasonable chance that it contains exon sequences. Subclones of the cosmids shown in FIG. 1 were used to screen Southern blots containing rodent DNA samples. A subclone of cosmid N5.66 (p 5.66-4) was shown to strongly hybridize to rodent DNA, and this clone was used to screen cDNA libraries derived from normal adult colon and fetal liver. The ends of the initial cDNA clones obtained in this screen were then used to extend the cDNA sequence. Eventually, 11 cDNA clones were isolated, covering 2314 bp. The gene detected by these clones was named TB1. Sequence analysis of the overlapping clones revealed an open reading frame (ORF) that extended for 1302 bp starting from the most 5' sequence data obtained (FIG. 2A). If this entire open reading frame were translated, it would encode 434 amino acids (SEQ ID NO:5). The product of this gene was not globally homologous to any other sequence in

the current database but showed two significant local similarities to a family of ADP, ATP carrier/translocator proteins and mitochondrial brown fat uncoupling proteins which are widely distributed from yeast to mammals. These conserved regions of TB1 (underlined in FIG. 2A) may define a predictive motif for this sequence family. In addition, TB1 appeared to contain a signal peptide (or mitochondrial targeting sequence) as well as at least 7 transmembrane domains.

A11

At column 15, line 10:

Because of the proximity of the MCC and APC genes, and the fact that both [am] <u>are</u> implicated in colorectal tumorigenesis, we searched for similarities between the two predicted proteins. Bourne has previously noted that MCC has the potential to form alpha helical coiled coils (Nature, Vol. 351, p. 188 (1991). Lupas and colleagues have recently developed a program for predicting coiled coil potential from primary sequence data (Science, Vol. 252, p. 1162 (1991) and we have used their program to analyze both MCC and APC. Analysis of MCC indicated a discontinuous pattern of coiled-coil domains separated by putative "hinge" or "sparer" regions similar to those seen in laminin and other intermediate filament proteins. Analysis of the APC sequence revealed two regions in the N-terminal domain which had strong coiled coil-forming potential, and these regions corresponded to those that showed local similarities with myosin and IF proteins on database searching. In addition, one other putative coiled coil region was identified in the central region of APC. The potential for both APC and MCC to form coiled coils is interesting in that such structures often mediate homo- and [hetero-oligomerization] <u>heterooligomerization</u>.

A12

At column 17, line 45:

We next examined three exons of the [A PC] <u>APC</u> gene. The three exons examined

A13

included those containing nt 822-930, 931-1309, and the first 300 nt of the most distal exon (nt 1956-2256). PCR and RNase protection analysis were performed as described in Kinzler et al. supra, using the primers underlined in Table I (SEQ ID NO:24-38). The primers for nt 1956-2256 were 5'-GCAAATCCTAAGAGAGAACAA-3' (SEQ ID NO:99) and 5'-GATGGCAAGCTTGAGCCAG-3' (SEQ ID NO:100).

A13

At column 17, line 54:

In 90 kindreds, the RNase protection method was used to screen for mutations and in an additional 13 kindreds, the PCR products were cloned and sequenced to search for mutations not detectable by RNase protection. PCR products were cloned into a Bluescript vector modified as described in T. A. Holton and M. W. Graham, Nucleic Acids Res. 19, 1156 (1991). A minimum of 100 clones were pooled and sequenced. Five variants were detected among the 103 kindreds analyzed. Cloning and subsequent DNA sequencing of the PCR product of patient P21 indicated a C to T transition in codon 413 that resulted in a change from arginine to cysteine. This amino acid variant was not observed in any of 200 DNA samples from individuals without FAP. Cloning and sequencing of the PCR product from patients P24 and P34, who demonstrated the same abnormal RNase protection pattern indicated that both had a C to T transition at codon 801 that resulted in a change from arginine (CGA) to a stop codon (TGA). This change was not present in 200 individuals without FAP. As this point mutation resulted in the predicted loss of the recognition site for the enzyme Taq I, appropriate PCR products could be digested with Taq I to detect the mutation. This allowed us to determine that the stop codon [co-segragated] cosegregated with disease phenotype in members of the family of P24. The inheritance of this change in affected members of the pedigree provides additional evidence for the importance of the mutation.

A14

At column 19, line 21:

In addition to these germline mutations, we identified several somatic mutations of MCC and APC in sporadic [CRC's] CRCs. Seventeen MCC exons were examined in 90 sporadic colorectal cancers by RNase protection analysis. In each case where an abnormal RNase protection pattern was observed, the corresponding PCR products were cloned and sequenced. This led to the identification of six point mutations (two described previously) (Kinzler et al., supra), each of which was not found in the germline of these patients (Table IIB).

At column 21, line 8:

Because patient 8214 showed [bray] only a 940 kb NotI fragment, she had not inherited the 1200 kb fragment present in the unaffected father's DNA. This observation suggests that he must be heterozygous for, and have transmitted, either a deletion of the L5.79 probe region or a variant NotI fragment too large to resolve on the gel system. As expected, the hybrid cell line HHW1159, which carries the paternal homolog, revealed no resolved Not fragment when probed with L5.79. However, probing of HHW1159 DNA with L5.79 following digestion with other enzymes did reveal restriction fragments, demonstrating the presence of DNA homologous to the probe. The father is, therefore, interpreted as heterozygous for a polymorphism at the NotI site, with one chromosome 5 having a 1200 kb NotI fragment and the other having a fragment too large to resolve consistently on the gel. The latter was transmitted to patient 3214.

At column 21, line 23:

When double digests were used to order restriction sites within the 1200 kb NotI fragment, L5.71 and L5.79 were [beth] both found to lie on a 550 kb NotI-NruI fragment and, therefore, on the same side of an NruI site in the 1200 kb NotI fragment. To obtain genomic representation of sequences present over the entire 1200 kb NotI fragment, we constructed a

library of small-fragment inserts enriched for sequences from this fragment. DNA from the somatic cell hybrid HHW141, which contains about 40% of chromosome 5, was digested with NotI and electrophoresed under pulsed-field gel (PFG) conditions; EcoRI fragments from the 1200 kb region of this gel were cloned into a phage vector. Probe Map30 was isolated from this library. In normal individuals probe Map30 hybridizes to the 1200 kb NotI fragment and to a 200 kb NruI fragment. This latter hybridization places Map30 distal, with respect to the locations of L5.71 and L5.79, to the NruI site of the 550 kb NotI-NruI fragment.

At column 24, line 44:

Two additional probes for the locus, YS-11 and YS-39, which had been ascertained by screening of a cDNA library with an independent YAC probe identified with MCC sequences adjacent to L5.71, were mapped into the deletion region. YS-39 was shown to be a cDNA identical in sequence to DP1. Partial characterization of YS-11 had shown that 200 bp of DNA sequence at one end was identical to sequence coding for the 19 kd protein of the ribosomal signal recognition particle, SRP19 (Lingelbach et al., supra). Hybridization experiments mapped YS-11 within [beth] both deletions. The sequence of this clone, however, was found to be complex. Although 454 bp of the 1032 bp sequence of YS-11 were identical to the GenBank entry for the SRP19 gene, another 578 bp appended 5' to the SRP19 sequence was found to consist of previously unreported sequence containing no extended open reading frames. This suggested that YS-11 was either a chimeric clone containing two independent inserts or a clone of an incompletely processed or aberrant message. If YS-11 were a conventional chimeric clone, the independent segments would not be expected to map to the same physical region. The segments resulting from anomalous processing of a continuous transcript, however, would map to a single chromosomal region.

At column 30, line 3:

DNA samples from both parents, from the patient's wife, and from their three children were examined. SSCP analysis of DNA from both of the patient's parents displayed the normal pattern of conformers for exon 7, as did DNA from the [patients's] patient's wife and one of his off-spring. The two other children, however, displayed the same new conformers as their affected father. Testing of the patient and his parents with highly polymorphic VNTR (variable number of tandem repeat) markers showed a 99.98% likelihood that they are his biological parents.

Remarks

The Amendments

The amendments to the specification correct clerical errors.

Reissue Declaration

The reissue declaration filed with the application inadvertently was missing the clause regarding "willful false statements" required by 37 C.F.R. § 1.68. Reissue declarations containing the required paragraph and executed by six of the named inventors accompany this amendment. We will forward the executed declarations of the remaining inventors as soon as they are received.

Offer to Surrender the Original Patent

The Office Action states that this reissue application was filed without the required offer to surrender the original patent. Assignees made the required offer on page 2 of the paper